



# Research Techniques Made Simple: Techniques to Assess Cell Proliferation

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Cell proliferation is commonly assayed in the laboratory for research purposes, but is increasingly used clinically to gauge tumor aggressiveness and potentially guide care. Therefore, both researchers and clinicians should have a basic understanding of techniques used to assess cell proliferation. Multiple cell proliferation assays exist, and the choice of method depends on the laboratory resources available, the types of cells/tissues to be studied, and the specific experimental goals. In this article, we identify four overarching categories of cell proliferation assays that signify various stages of the cell cycle: nucleoside-analog incorporation, cell cycle-associated protein detection, use of cytoplasmic proliferation dyes, and indirect measures of cell proliferation. Each method has strengths and limitations that should guide the dermatology investigator's choice of assay.

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## INTRODUCTION

Cell proliferation is defined as an increase in cell number secondary to cell growth and division (reviewed in [Schafer, 1998](#)). Assessing cell proliferation is a cornerstone of basic, translational, and clinical research and of clinical medicine. Given this pervasiveness, it is important for the clinical dermatologist and dermatology researcher alike to have a basic comprehension of cell proliferation and the assays most commonly used to measure it.

A number of methods exist to measure cell proliferation, and they vary in regard to which phase of cellular growth and division they assay, the equipment and expertise required, whether additional studies can be performed in parallel or in series, and what types of cells/tissues can be studied by that assay. To simplify this topic, this article outlines the principal approaches used to assess cell proliferation based on three aspects of cell division: nucleoside-analog incorporation during DNA synthesis, cell cycle-associated proteins, and cytoplasmic proliferation dyes. A fourth section briefly reviews indirect methods of assessing cell proliferation via cell counting and viability and metabolic activity assays.

## THE CELL CYCLE

Cellular proliferation results from progression through the cell cycle ([Figure 1](#)). The cell cycle has two major phases: interphase and mitosis (reviewed in [Schafer, 1998](#)). A cell spends most of its life in interphase, which is divided into three stages: Gap 1 (G<sub>1</sub>), Synthesis (S), and Gap 2 (G<sub>2</sub>). During interphase, the cell is growing and preparing for division. Cellular division occurs during mitosis, or (M) phase, which actually consists of both mitosis and cytokinesis. Mitosis refers to division of the nucleus resulting in equal separation of chromosomes and is subdivided into phases: prophase, prometaphase, metaphase, anaphase, and telophase. Cytokinesis is the equal division of the cell membrane, cytoplasm, and organelles. M phase results in two

## BENEFITS

- Commonly utilized in laboratory research and increasingly in clinical practice.
- Broad range of assays available to assess various stages of the cell cycle.
- Some assays can be performed in conjunction with other stains, and some techniques maintain viable cells for follow-up experimentation.
- Many methods are technically straightforward and utilize commonly available equipment.

## LIMITATIONS

- Some assays require specialized equipment and/or materials.
- Available sample(s) may be incompatible with certain techniques (eg, cytoplasmic dyes and thymidine incorporation cannot be used on formalin-fixed clinical skin specimens).
- Some assays are lethal to cells, so further experimentation cannot be performed.

daughter cells identical to their parent cell. A fourth stage, Gap zero (G<sub>0</sub>), describes resting cells and cells that rarely or never divide.

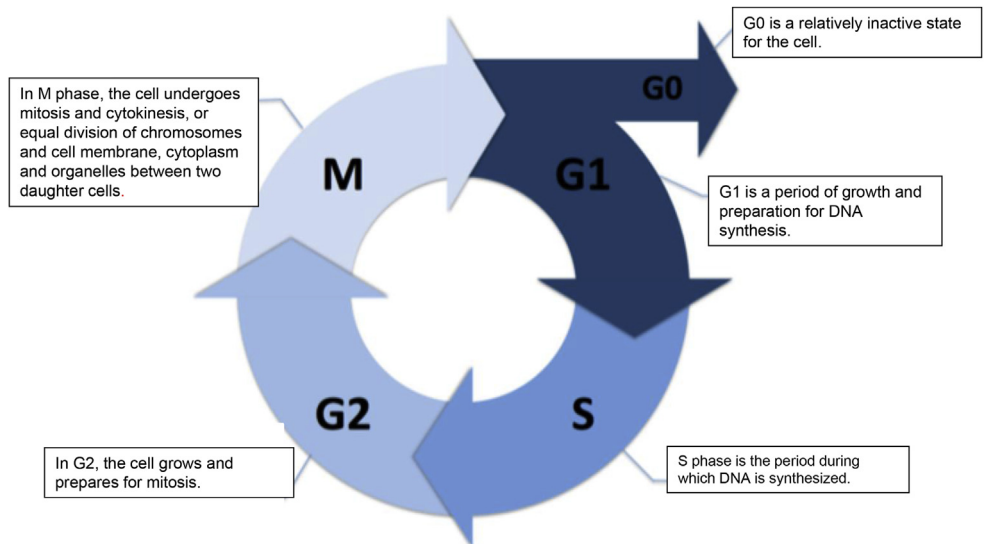
The stages of the cell cycle can be identified based on specific characteristics. For example, nucleosides are incorporated into replicating DNA exclusively during S phase, and histone protein H3 is only phosphorylated during M phase. Many proliferation assays take advantage of these unique characteristics of each cell cycle phase.

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**Figure 1. Schematic of the cell cycle depicting the phase detected by different proliferation assays.** G<sub>0</sub> is a relatively inactive state for the cell.

G<sub>1</sub> is a period of cell growth and preparation for DNA synthesis, or the S phase. Cell growth continues during G<sub>2</sub> as the cell prepares for mitosis (M) phase. M phase consists of equal division of chromosomes and cytoplasmic components (termed cytokinesis) between two daughter cells. Cytoplasmic proliferation dyes are internalized during all phases of the cell cycle including G<sub>0</sub>, but proliferation is only appreciated once cytokinesis has occurred and the fluorescence intensity of the dye is halved in the daughter cells. Ki-67 is expressed during active phases of the cell cycle (G<sub>1</sub>-M). Nucleoside-analog incorporation assays (ie, [<sup>3</sup>H]TdR and BrdU) and proteins such as PCNA are specific to the S phase. BrdU, 5-bromo-2'-deoxyuridine; [<sup>3</sup>H]TdR, tritiated thymidine; PCNA, proliferating cell nuclear antigen.



### NUCLEOSIDE-ANALOG INCORPORATION ASSAYS

During the S phase of the cell cycle, genome replication, DNA polymerases incorporate nucleosides (eg, deoxyadenosine, deoxyguanosine, deoxycytidine, and thymidine) into new strands of DNA. Nucleoside-analog incorporation assays introduce chemically or radioactively labeled nucleosides into the sample of interest, which are incorporated into newly synthesized DNA during S phase.

A classic example of this strategy is the tritiated thymidine ([<sup>3</sup>H]TdR) incorporation assay whereby excess radiolabeled thymidine is added to cell cultures and allowed to incubate for multiple days. Excess [<sup>3</sup>H]TdR is then washed away and incorporated. [<sup>3</sup>H]TdR is measured using a liquid scintillation counter. The assay can be performed in vitro or ex vivo, but not in vivo. This method quantifies overall division compared with a control and is commonly regarded as reliable and accurate. Assays should be run in triplicate and using varying numbers of cells to generate a proliferation curve (Figure 2a). Drawbacks are that radioactive reagents must be handled and disposed of with caution, thymidine is measured by the scintillation counter per well of cells rather than per individual cell so the assay reveals nothing about an individual cell's division history, and no additional assays can be performed with or after [<sup>3</sup>H]TdR incorporation (it is an endpoint assay) because the assay extracts DNA from cells that are then washed away in the process. A common use for [<sup>3</sup>H]TdR incorporation is to assess T-cell proliferation, for example, in response to therapeutic dendritic cells, as shown in Figure 2a (Divito et al., 2010).

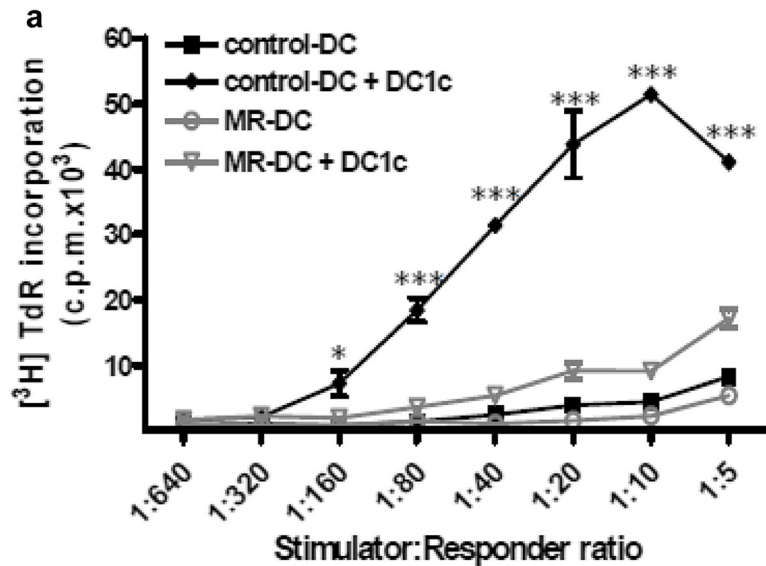
Another common nucleoside-analog incorporation assay employs 5-bromo-2'-deoxyuridine (BrdU), also a thymidine analog. Incorporated BrdU is detected by a BrdU-specific monoclonal antibody that may be bound directly to a fluorescent tag or measured indirectly via a secondary antibody. Fluorescence can then be measured via a flow cytometer or fluorescence microscopy (Figure 2b). Alternatively,

chromogenic tagging (colored, rather than fluorescent) can be performed that requires only a standard light microscope for inspection. An added benefit of the assay is that DNA can be "captured" via the antibody (ie, bound by the antibody, then purified by immunoprecipitation, and subsequently sequenced). Further, BrdU can be used not only in vitro but also in vivo; it can be injected into an animal or added to the animal's drinking water and ingested. BrdU is stably incorporated into DNA, so it persists for several months. Additional stains can be included in this assay, for example, to identify the particular type of cell that is proliferating. Lastly, there is no handling of radioactive material. Two negatives of this method are that it is an endpoint assay because staining requires cell membrane permeabilization and fixation, and it cannot identify cells that have undergone numerous rounds of division (reviewed in Lyons et al., 2013).

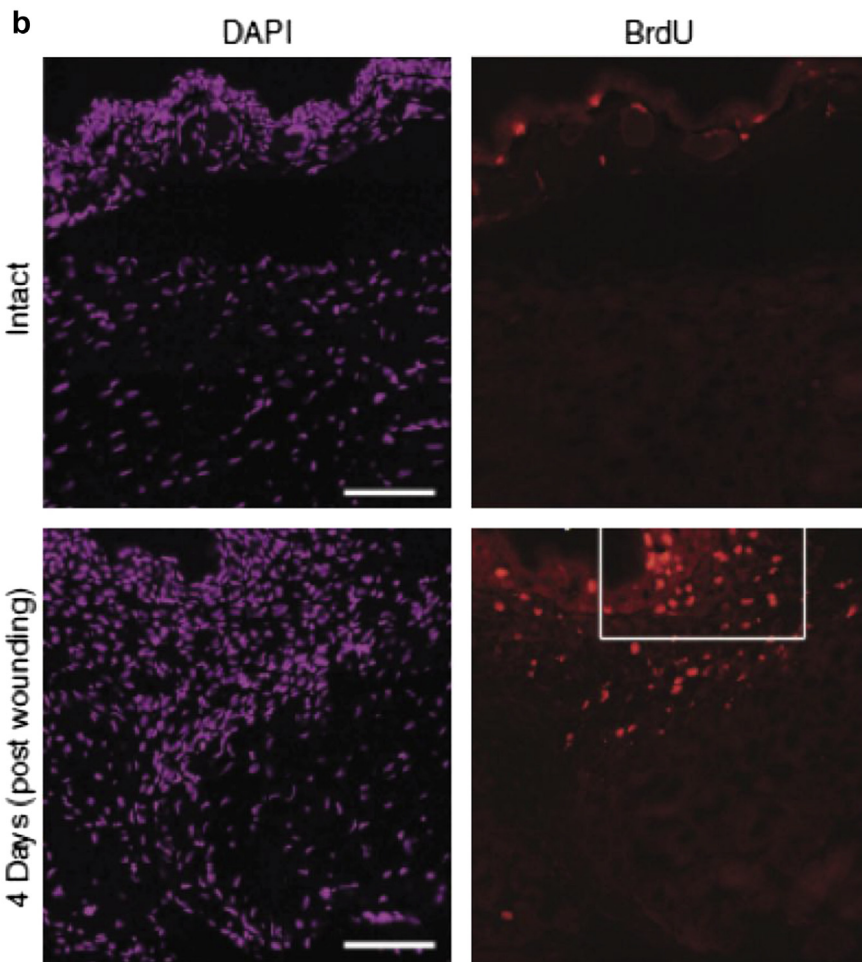
### CELL CYCLE-ASSOCIATED PROTEIN ASSAYS

As cells progress through the cell cycle, phase-specific proteins are generated and can be detected using antibodies. Example phase-specific proteins include topoisomerase II alpha, phosphorylated-histone H3, and proliferating cell nuclear antigen, though there are many others. Topoisomerase II alpha expression begins in late S phase and peaks in the G<sub>2</sub> and M phases (Woessner et al., 1991). Histone H3 becomes phosphorylated only during the M phase of the cell cycle (reviewed in Hans and Dimitrov, 2001). Proliferating cell nuclear antigen expression increases during late G<sub>1</sub> and peaks during S phase (Kurki et al., 1987). The most commonly assayed cell cycle-associated protein is Ki-67, although it does not actually allow delineation of the different cell cycle phases because it is expressed in G<sub>1</sub>, S, G<sub>2</sub>, and M, but absent in the resting phase, G<sub>0</sub>.

The major advantage of assaying cell cycle-associated proteins is that multiple techniques can be used: formalin-fixed paraffin-embedded and frozen tissue samples by



**Figure 2. Example data from nucleoside-analog incorporation assays.** (a) A tritiated thymidine ( $[^3\text{H}]\text{TdR}$ ) incorporation assay was performed to assess T-cell proliferation in response to treatment with therapeutic dendritic cells (DCs). Different ratios of irradiated dendritic cells (stimulator) (incapable of proliferating) to T cells (responder) were incubated together for 3 days; then  $[^3\text{H}]\text{thymidine}$  was added to cultures and measured by liquid scintillation counter to generate proliferation curves shown. \*\*\* represents statistical significance between groups. Reprinted with permission from [Divito et al. \(2010\)](#). (b) Fluorescent microscope images demonstrating increased cell proliferation (BrdU+, red) 4 days into wound healing in skin. Dapi images highlight the location of cell nuclei (purple). Reprinted with permission from [Yokoyama et al. \(2011\)](#).

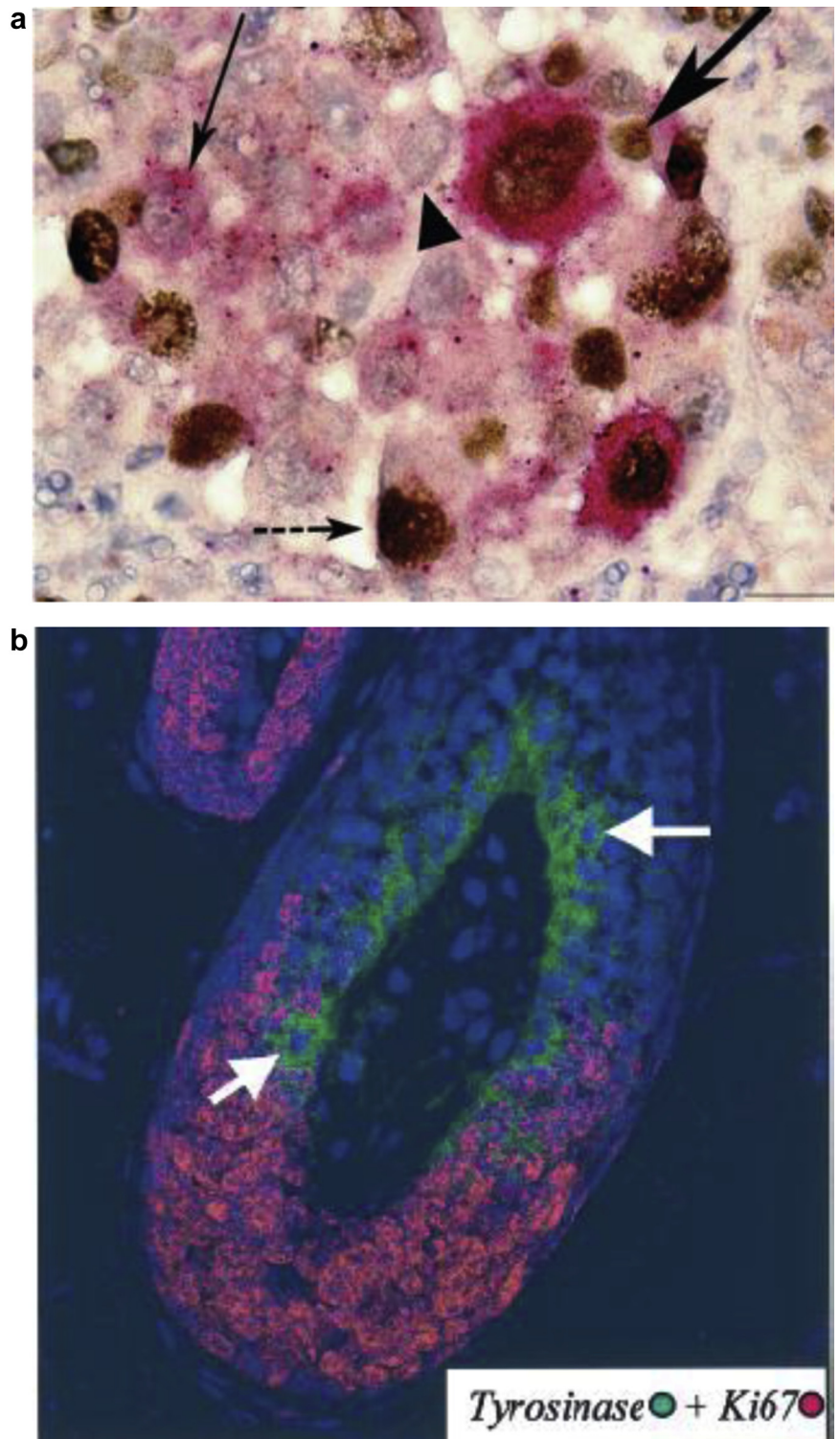


microscopy, single cell suspensions by flow cytometry, and cell lysates by western blot (though the last will provide only information on a collection of cells rather than an individual cell). This means that these markers can be used to assay proliferation in patient specimens, provided the tissue was fixed immediately after harvesting. Ki-67 is increasingly

used to detect proliferating cells in clinical melanoma specimens ([Gimotty et al., 2005](#)). [Figure 3a](#) depicts immunohistochemical staining of Ki-67 together with melanocyte differentiation antigen in a patient's melanoma specimen ([Aris et al., 2011](#)). [Figure 3b](#) shows immunofluorescence staining Ki-67 to assess melanocyte proliferation during hair follicle



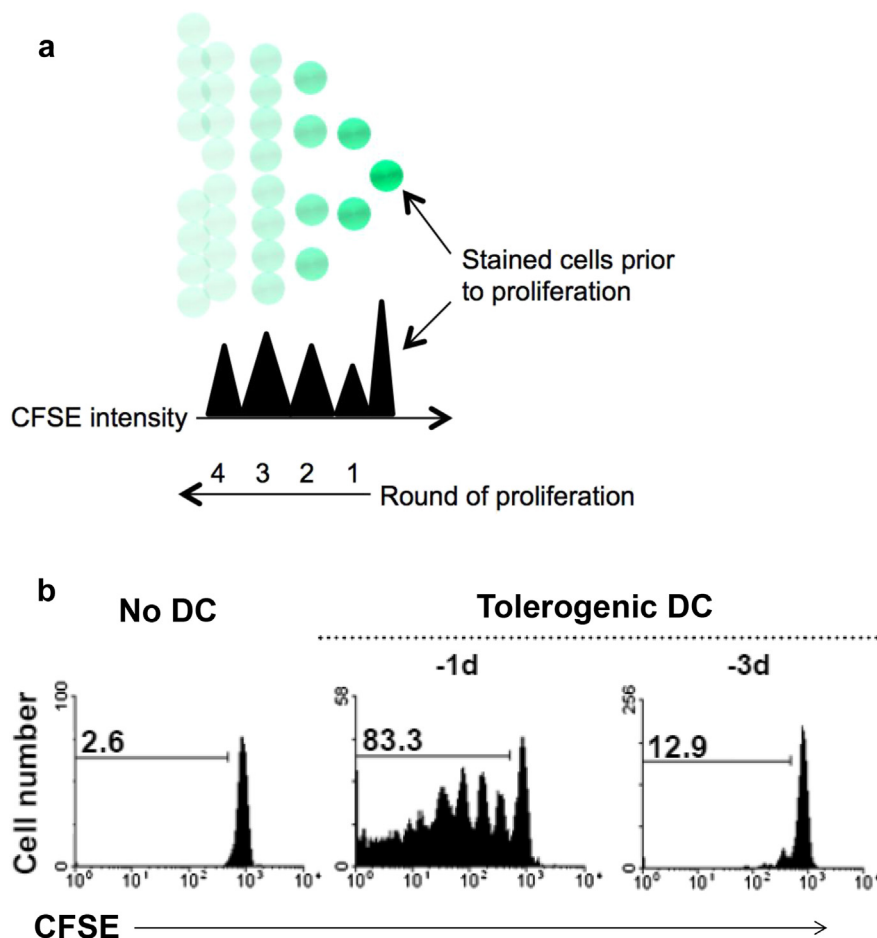
**Figure 3. Example staining and microscopy of Ki-67.** (a) Chromogenic staining detected via light microscopy of Ki-67 (brown) and melanocyte differentiation antigen, MD-Ag (red) in melanoma. Arrows depict different staining patterns (Ki-67<sup>+</sup>MD-Ag<sup>-</sup>, Ki-67<sup>+</sup>MD-Ag<sup>+</sup>, Ki-67<sup>-</sup>MD-Ag<sup>+</sup>, and Ki-67<sup>-</sup>MD-Ag<sup>-</sup>). Reprinted with permission from [Aris et al. \(2011\)](#). (b) Fluorescent microscopy depicting Ki-67 expression (red) and tyrosinase (green) in a hair follicle during development. Nuclei counterstained in blue (TO-PRO3 staining). Reprinted with permission from [Botchkareva et al. \(2003\)](#).



development ([Botchkareva et al., 2003](#)). A second advantage is that researchers can easily stain multiple cell cycle-associated proteins at one time to ascertain a cell's stage of proliferation. A disadvantage is that no subsequent assays can be performed with cell cycle-associated protein staining because of fixation and permeabilization of the cell's membranes.

#### CYTOPLASMIC PROLIFERATION DYES

A third approach for assessing cell proliferation utilizes cytoplasmic proliferation dyes that are fluorescent chemicals that permeate the cell membrane and covalently bind to cellular cytosolic components (reviewed in [Quah and Parish, 2012](#)). After each cell division, the dye is evenly distributed between the two daughter cells (as a result of



**Figure 4. Schematic and example data of carboxyfluorescein diacetate succinimidyl ester (CFSE) cytoplasmic proliferation dye assay.** (a) Schematic showing decreased CFSE fluorescence intensity exhibited by daughter cells after each successive round of cell division. Each black peak reflects one round of division, and the taller the peak, the more cells underwent division during that round. (b) Flow cytometry histograms demonstrating T-cell proliferation in vivo when intravenously injected into mice 1 or 3 days after that mouse was treated with tolerogenic dendritic cells or not (no DC). Reprinted with permission from Divito et al. (2010).

cytokinesis), which then exhibit half the fluorescence intensity of their parent cell (Figure 4a) (reviewed in Quah and Parish, 2012). Cytoplasmic proliferation dyes can be used both in vitro and in vivo. For in vivo use, single cell suspensions are stained with a cytoplasmic dye and then injected into an animal. The cells can then later be harvested from the animal and assayed to determine whether

the cells proliferated in vivo. When cytoplasmic dyes are used to assess proliferation, flow cytometry is the assay of choice as it allows visualization of each round of division (Figure 4). Although cytoplasmic dyes can be viewed under microscopy, microscopy does not easily allow quantification of dye and therefore is not typically a reliable means of measuring division.

**Table 1. Summary of commonly employed cell proliferation assays including associated cell-cycle phase, equipment, and example recommended applications for each assay**

Approach	Assay	Cell-cycle phase	Major equipment	Example recommended application(s)
Nucleoside-analog incorporation	Tritiated thymidine	S	Liquid scintillation counter	Cell culture, mixed lymphocyte reactions
Nucleoside-analog incorporation	5-Bromo-2'-deoxyuridine	S	Flow cytometer, light microscope, fluorescence microscope	In vivo for the study of proliferation over time in animals
Cell cycle-associated protein	Ki-67	G1, S, G2, M	Flow cytometer, light microscope, fluorescence microscope	Histology
Cell cycle-associated protein	Phosphorylated-histone H3	M	Flow cytometer, light microscope, fluorescence microscope	Histology
Cell cycle-associated protein	Proliferating cell nuclear antigen	S	Flow cytometer, light microscope, fluorescence microscope	Histology
Cytoplasmic proliferation dye	Carboxyfluorescein diacetate succinimidyl ester	Cytokinesis	Flow cytometer	In vivo proliferation of adoptively transferred stained cells, mixed lymphocyte reactions
Cytoplasmic proliferation dye	Cell trace violet	Cytokinesis	Flow cytometer with a 405-nm laser	In vivo proliferation of adoptively transferred stained cells, mixed lymphocyte reactions

The classic cytoplasmic dye used in proliferation assays is carboxyfluorescein diacetate succinimidyl ester (CFSE), which is a green fluorescent dye. Because neither CFSE staining nor flow cytometry is an endpoint assay, dyed cells can be sorted and used in further experimentation. One word of caution with CFSE: after enough rounds of division, CFSE fluorescence will no longer be detectable above nonlabeled cells. With optimization, however, the CFSE technique can detect beyond seven rounds of proliferation (Quah and Parish, 2012). A frequent use of CFSE is to analyze T-cell proliferation. For example, Seneschal et al. (2014) used CFSE staining to demonstrate in vivo that dermal dendritic cells stimulate transgenic T-cell proliferation in response to vaccination, and Divito et al. (2010) used CFSE to assess T-cell proliferation in response to therapeutic dendritic cells (Figure 4b).

A drawback of CFSE is that it utilizes the green fluorescent channel, thereby preventing inclusion of additional stains that also require that channel (ie, fluorescein, green fluorescent protein and fluorescein isothiocyanate) (reviewed in Quah and Parish, 2012). Newer commercially available stains such as CellTrace Violet (ThermoFisher Scientific, Cambridge, MA) are gaining popularity because they fluoresce under a different wavelength of light, thus making available the fluorescein channel for other stains (Quah and Parish, 2012). CellTrace Violet does, however, require a blue/violet laser that may not be available on all detection equipment.

#### INDIRECT MEASURES OF CELL PROLIFERATION: CELL COUNTING, VIABILITY, AND METABOLIC ACTIVITY ASSAYS

Information regarding cell proliferation may be gleaned from cell counting alone or via cell viability stains or assays. The most commonly employed viability stain is trypan blue that is excluded from living cells but absorbed by dead cells (dead cells thus appear blue under light microscopy). Metabolic activity assays use enzymatic activity as a marker for cell viability. The enzyme(s) tested produce a colored or fluorescent product that can be easily measured by a plate reader. Example metabolic activity assays are the tetrazolium assay, resazurin reduction assay, and protease activity assay (reviewed in Riss et al., 2013). It is important for the reader to understand that none of these methods directly assess cell proliferation. For example, cell counting may suggest that cell proliferation has occurred if there is an increase in the number of cells. However, an alternative explanation could be that cell death decreased. Similarly, an increase in enzymatic activity in a group of cells could indicate increased number of cells due to proliferation, or could reflect increased metabolic activity of nonproliferating cells. Therefore, when interpreting studies using these techniques (like all studies), the reader should ask him or herself, “could anything else account for the observed data” and if so, “do either the presented experimental controls or further experiments address these alternative possibilities?”

#### CONCLUDING REMARKS

Table 1 summarizes the most commonly employed cell proliferation assays, including which cell cycle phase the technique assesses and the major equipment needed for that technique. Table 1 also includes recommendations regarding

which assay may be most appropriate for a particular experimental application. However, as stated in the introduction, choice of a particular assay is highly individualized based on the experimental objectives, available equipment, and sample type. Clinically, we expect that staining for cell proliferation markers to assess tumor aggressiveness and guide clinical care will likely become standard of care going forward, particularly in the setting of melanoma, where absence/presence of mitotic figures is already part of melanoma clinical staging. This article provides a framework for the investigator and clinician to understand cell proliferation assays, but the reader should know that modifications and

#### MULTIPLE CHOICE QUESTIONS

1. Which of the following is NOT used for assessing cell proliferation?
  - A. Cytoplasmic proliferation dyes
  - B. Cell cycle-associated proteins
  - C. Nucleoside-analogs incorporated during DNA synthesis
  - D. Bioassay-guided fractionation
2. Which of the following methods uses radioactive material?
  - A. Tritiated thymidine incorporation
  - B. Cell counting
  - C. 5-Bromo-2'-deoxyuridine incorporation
  - D. Carboxyfluorescein diacetate succinimidyl ester dye
3. In which of the following phases of the cell cycle is Ki-67 expression absent?
  - A. G<sub>0</sub>
  - B. G<sub>1</sub>
  - C. S
  - D. G<sub>2</sub>
4. Which proliferation assay can be used on patient samples that are formalin-fixed and paraffin embedded?
  - A. CFSE labeling
  - B. Tritiated thymidine incorporation
  - C. Ki-67 staining
  - D. BrdU incorporation
5. Which assay can be used to measure proliferation in vivo in an animal over time? Which assay provides a snapshot of proliferation at the time of tissue harvest?
  - A. Tetrazolium salt assay, Ki-67 staining
  - B. Histone H3 staining, CFSE labeling
  - C. BrdU incorporation, Ki-67 staining
  - D. Ki-67, BrdU incorporation

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optimization of the above assays are ongoing and new techniques are constantly under development.

#### CONFLICT OF INTEREST

The authors state no conflict of interest.

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#### SUPPLEMENTARY MATERIAL

Supplementary material is linked to this paper.

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